Attorney Docket No. FP0602.1 US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application: Volkmar Guenzler-Pukall, et al.	Confirmation No.: 5297
Application No.: 10/729,704	Examiner: Roy R. Teller
Filed: 4 December 2003	Group Art Unit: 1654
Title: TREATMENT OF DIABETES	Customer No.: 041385

FILED ON: 13 OCTOBER 2010

DECLARATION OF INVENTOR
UNDER 37 C.F.R. § 1.132

Sir:

I, Volkmar Guenzler-Pukall, M.D., being duly warned that willful false statements and the like are punishable by fine or imprisonment or both (18 U.S.C. § 1001), and may jeopardize the validity of the patent application or any patent issuing thereon, state and declare as follows:

1. All statements herein made of my own knowledge are true, and statements made on information or belief, are believed to be true and correct.

2. I received a diploma in chemistry (Masters equivalent) from the University of Marburg, Germany and a Dr. med. (M.D., Ph.D. equivalent) from the Universities of Marburg and Mainz, Germany. A copy of my curriculum vitae is attached hereto as **Exhibit A**. I have performed or directed research and development work on prolyl hydroxylase and their inhibitors for approximately 35 years, including work on hypoxia inducible factor (HIF) hydroxylase inhibitors. Currently, the development of novel HIF hydroxylase inhibitors and their uses is a focus of my work at FibroGen, Inc., San Francisco, CA.

3. I am a named inventor on the above-referenced patent application. The invention of this application provides for the first time methods for treating diabetes, treating hyperglycemia and reducing blood glucose levels in a subject by administering a heterocyclic carbonyl glycine compound which inhibits a HIF hydroxylase. Inhibition of HIF hydroxylase activity increases the expression of genes involved in glucose transport and utilization resulting in a reduction of blood glucose levels. The reduction in blood glucose levels is a primary means of treating diabetes and hyperglycemia.

4. I have read and am familiar with the contents of this patent application. In addition, I have read the Office Action, mailed September 23, 2009, received in the present case. It is my understanding that the Examiner does not believe that the claimed method for treating diabetes or hyperglycemia by administering a heterocyclic carbonyl glycine compound which inhibits a HIF hydroxylase is sufficiently enabled because the Examiner is skeptical that a person skilled in the art, upon reading the specification, would be able to make or use the invention to the extent of the scope of the claims.

5. This declaration is provided to offer evidence that demonstrates that a person of ordinary skill in the art upon reading the recited claims would readily understand that the treatment methods encompass the use of compounds that meet two limitations: a structural limitation, i.e., heterocyclic carbonyl glycine compounds, and a functional limitation, i.e., inhibit HIF hydroxylase. This person would readily understand what is meant by the term "heterocyclic carboxy glycine" and would further know that each exemplified quinoline-2-carboxamide, isoquinoline-3-carboxamide and 3-hydroxypyridine carbonyl glycine meets the definition of a heterocyclic carboxy glycine compound. Therefore, a person of skill would know that the exemplified compounds meet the structural limitation.

Further, a person of ordinary skill would also know that the exemplified compounds meet the functional limitation, because they are demonstrated to be inhibitors of HIF hydroxylase. Additionally, a person of skill would recognize that the exemplified compounds are representative of their respective families of HIF hydroxylase inhibitors

disclosed in the cited patents and that these other family members would share the ability of the exemplified compounds to decrease blood glucose levels based on their common structural and functional characteristics. Furthermore, a person of skill would expect that any other compound that meets the two limitations of being a heterocyclic carbonyl glycine that inhibits HIF hydroxylase would also decrease blood glucose levels, because this compound will operate by the same mechanism as the exemplified compounds, through the inhibition of HIF hydroxylase.

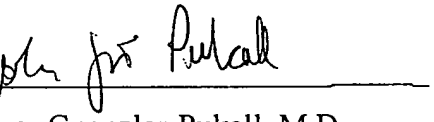
6. A person of skill would also readily understand the disclosed screening methodologies that allow one to identify other heterocyclic carbonyl glycines with the ability to inhibit HIF hydroxylase. Contrary to the Examiner's assertions, the assays and experimental procedures are well within the ability of a person of ordinary skill. The performance of such screening assays is routine in the pharmaceutical research and development fields. The analysis of screening data is unambiguous and straight forward making it relatively easy to identify compounds that inhibit HIF hydroxylase activity. In my opinion, the performance of a routine screening assay does not represent undue experimentation regardless of the number of compounds screened.

7. Additionally, I attest that a person of ordinary skill would also understand the teaching in the application that heterocyclic carbonyl glycine HIF hydroxylase inhibitors, through the increase in cellular HIFα concentration, cause an up-regulation of the expression of glucose transport and utilization genes. A person of skill would readily understand the usefulness of the increased expression of these glucose transport and utilization genes in the treatment of conditions that feature elevated blood glucose levels such as diabetes and hyperglycemia. Given the direct correlation between the inhibition of HIF hydroxylase activity, the increase in cellular HIFα concentration, the increase in glucose transport and utilization gene expression and the resultant reduction in blood glucose levels, a person of skill would readily understand that all known heterocyclic carbonyl glycine HIF hydroxylase inhibitors and those identified using any one of the disclosed screening assays can be used to practice the invention encompassed by the recited claims.

8. In summary, we have obtained clear evidence through our research that heterocyclic carbonyl glycine HIF hydroxylase inhibitors increase the expression of genes involved in glucose transport and utilization. Further, we found that the increased gene expression leads to reduced blood glucose levels. This discovery is useful in treating conditions that feature elevated blood glucose levels, such as diabetes and hyperglycemia. Since all heterocyclic carbonyl glycine compounds that inhibit HIF hydroxylase are expected to share the ability to lower blood glucose levels, it is my scientific opinion that the claimed methods for treating diabetes and hyperglycemia and reducing blood glucose are fully enabled.

Respectfully submitted,

Dated: 12 October 2010



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Education:

Diploma in chemistry (Masters equivalent), University of Marburg, Germany (1978)
Dr. med. (M.D., Ph.D. equivalent), Universities of Marburg and Mainz, Germany (1985)

Professional Experience:

February 1999 – present: FibroGen, Inc, 409 Illinois Street, San Francisco, CA 94158

Presently held position: Senior Director of Clinical Research
Previous position: Director of Preclinical Research (1999-2005)

Responsible for Clinical Development of HIF stabilizers in Europe, 2005-2007; Leader of the CTGF Clinical Research program, FibroGen, Inc., San Francisco, CA., USA (2007-present)

Clinical Experience and Responsibilities (3/2005 – present):

- Supervision of multidisciplinary teams of up to 15 members, including biostatistics, data management, medical and operational staff
- Design, planning and execution of multiple Phase 1 (healthy volunteers and patients) and Phase 2 studies
    - Chronic kidney disease with several therapeutic agents (anti-anemia small molecules and the anti-fibrotic therapeutic monoclonal antibody FG-3019)
    - Cancer and related indications (pancreatic cancer, MDS, chemotherapy induced anemia)
    - Cytoprotection
    - Systemic sclerosis
    - Hepatitis B
- Multinational experience (US, Europe, Hong Kong)
- Development of Target Product Profiles and Clinical Development Plans
- Protocol design including translational aspects, incorporating feed-back from Scientific Advisory Boards and consultants
- Medical monitoring (Data review, protocol exceptions, SAE review in support of Drug Safety)
- Start-up activities (CRO selection and procurement, site selection, site initiation and training, Investigator's Meetings)
- Support of Data Management (Implementation of IVRS and IWRS systems, implementation of EDC systems including edit checks and UAT, TFL review, query resolution, coding review)

- Supervision of ancillary study documents (Investigator's Brochure, Study Reference Manual, Safety Plan, Medical Monitoring Plan, CRF Completion Guidelines, Communication Plan, DMC charter, Statistical Analysis Plan, Study Report)
- Support of Regulatory activities (IND filings, annual updates, safety summaries)
- Publication and presentation at scientific meetings

Scientific Preclinical Responsibilities (1999-2004):

- Project Leader of a team of up to 25 members in the areas of fibrotic diseases (with focus on fibrosis of the heart and skin)
- Initiation of a new therapeutic area that raised > $ 500 million in licensing fees (HIF stabilizing small molecules; anemia, cytoprotection)
- Nomination of clinical candidate compounds (anemia, cytoprotection)
- Management of collaborations with academic researchers in the USA, Germany, the Netherlands, the UK, and Australia
- Management of contract research
- Scientific publications and presentations
- SBIR grant applications
- Support of patent applications, evaluation of acquisitions

Operational Responsibilities:

- Budgeting
- Recruitment (involvement up to the level of VP)
- Performance evaluations
- Internal scientific audits, assay validation
- Temporary responsibilities as Acting Head of the Chemistry and Pharmacology Departments

Alliance management (1998-present):

- Astellas (anemia): Member of a team selection of a partner amongst > 10 Japanese pharmaceutical companies, negotiation, regular meetings and education after the strategic alliance was formed, with focus on medical/scientific questions
- ICI/Astra Zeneca (fibrosis): regular meetings and reports after the strategic alliance was formed, with focus on medical/scientific questions
- Selection of a contract manufacturer for anemia clinical candidate compounds (US, Switzerland, France, Germany; site visits, including assessment of GMP compliance)
- Multiple meetings and partnering discussions with major US and global pharmaceutical companies, for both anemia and anti-fibrosis programs
- Evaluation of the biotech landscape in Germany in preparation of a German subsidiary (VC contacts for funding, discussions with local government officials)
- Evaluation of intellectual property portfolios (anemia, HIF stabilizers, imaging)

- Evaluation of acquisition opportunities (chemical and biological companies; imaging and medical devices)
- Evaluation of in-licensing opportunities (scientific and clinical review)
- Management of multiple scientific collaborations, overseas and in the US

August 1998- February 1999: Consultant, FibroGen Inc

January 1987-July 1998
Senior scientist and "Leitender Angestellter" (Scientist with managenment responsibilities) at Hoechst AG / Hoechst Marion Roussel Deutschland GmbH, Frankfurt

Responsibilities:

- Management of a scientific laboratory with up to 8 direct reports. Research in the areas of fibrotic diseases with focus on liver and lung, as well as obesity / leptin
- Translational support of the clinical development of two antifibrotics (HOE 077 und 277) to Phase 2
- Interaction with health authorities including the FDA
- Development of immunological diagnostics (RIAs) of extracellular matrix proteins (Type IV collagen and laminin laboratory assays, RIAgnost PIIIP c.t. (Behringwerke, Marburg), commercial assay)
- Establish assays in HTS format, assay validation
- Plan and evaluate pharmacological models
- Biochemical and protein chemical analysis
- Protein purification and production of antibodies
- Molecular design
- Participation in the development of patent strategies
- Radiation safety officer
- Performance evaluation

Alliance management:

- Multiple meetings and partnering discussions with major US and global pharmaceutical companies (anti-fibrosis program)
- Establish and monitor academic collaborations in the USA, Germany, the Netherlands, France, and the Czech Republic
- Evaluation of in-licensing opportunities (scientific and clinical review)

Previous Positions:

- Post-Doc at the Department of Medical Biochemistry, University of Oulu, Oulu, Finnland (1985-1987)
- Medical residency at the Horst – Schmidt – Kliniken, Wiesbaden; Internal medicine, general surgery, surgery of trauma patients, dermatology
- Research scientist , Department of Pediatrics, Johannes – Gutenberg – University Mainz (1981-1984)
- Research scientist, Department of Internal Medicine, Philipps – University Marburg (1978-1979)

Honors and awards:

- Scientific Advisor to the German Association of Thalidomide Victims (Bundesverband Contergangeschädigter); representation of the Association in mass media (press, television)
- Scholarship from the "Studienstiftung des Deutschen Volkes", 1972-1976
- Long term fellowship from the European Molecular Biology Organization, 1985-1987
- Visiting Docentship, University of Oulu, Finland 1989
- Shareholder Award, FibroGen 2004
- Executive Recognition Award, FibroGen 2006

Languages:

- German (primary language)
- English
- Conversant in Japanese (Level 3 JLPT, 2005); reading abilities; translations (patents, scientific literature, publications)
- Reading abilities in Spanish and French
- Basic Finnish

Other activities:

- Extensive experience in the evaluation of scientific work of others
- Supervision of thesis work
- Evaluation of thesis work up to docent level

Patents and Patent Applications:

Hoechst AG: Co-inventor of > 30 individual filings, small molecules and diagnostics; cited as one of the principal inventors of Hoechst AG in a published analysis of the IP portfolio (P. Steele, Company Profile - Hoechst: analysis of patenting 1991 - 1994, Exp. Opin. Ther. Patents 4, 1309-1318 (1994))

FibroGen, Inc: Co-inventor of the following patents and patent applications:

1. Novel Nitrogen-Containing Heteroaryl Compounds And Methods of Use Thereof; Publication info: US2007185159 - 2007-08-09

2. Stabilization of hypoxia inducible factor (HIF) alpha; Publication info: US2006270699 - 2006-11-30

3. Use of HIF alpha stabilizers for enhancing erythropoiesis; Publication info: CN1816327 - 2006-08-09

4. Nitrogen-containing heteroaryl compounds and their use in increasing endogenous erythropoietin Publication info: CN1816527 - 2006-08-09

5. Methods of increasing endogenous erythropoietin (EPO) Publication info: US2006183695 - 2006-08-17

6. Treatment of diabetes; Publication info: CN1720052 - 2006-01-11

7. Fat regulation; Publication info: NO20053116 - 2005-09-05

8. Tissue remodeling and vascularization; Publication info: WO2005034929 - 2005-04-21

9. Cytoprotection; Publication info: WO2005007192 - 2005-01-27

Scientific publications:

1. H. Hanauske-Abel and V. Günzler, Inhibition of human prolyl hydroxylase as common biochemical denominator of the non-sedative effects of thalidomide in man. Z. Naturforsch. 32c, 241-248 (1977)

2. H.M. Hanauske-Abel and V. Günzler, A stereochemical concept for the catalytic mechanism of proly1 hydroxylase. Applicability to classification and design of inhibitors. J. theor. Biol. 94, 421-455 (1982)

3. V. Günzler, K. Majamaa, H.M. Hanauske-Abel, and K.I. Kivirikko, Inhibition of prolyl 4-hydroxylase by structure analogs of 2-oxoglutarate. Collagen Rel. Res. 3, 71 (1982)

4. V. Günzler, R.E. Schopf, H.M. Hanauske-Abel and H. Schulte-Wissermann, Transglutaminase and polyamine dependence of effector functions of human immunocompetent cells. The effect of specific inhibitors on lymphocyte proliferation and granulocyte chemiluminescence. FEBS Lett. 150, 390-396 (1982)

5. Amthauer, V. Günzler, S.S. Hafner, and D. Reinen, The distribution of Fe^{2+} and Ga^{3+} between octahedral and tetrahedral sites in garnets, $Y_3(Fe,Ga)_5O_{12}$, at different temperatures. Z. Kristallogr. 161, 167-186 (1982)

6. K. Majamaa, H.M. Hanauske-Abel, V. Günzler, and K.I. Kivirikko, The 2-oxoglutarate binding site of prolyl 4-hydroxylase. Identification of distinct subsites and evidence for 2-oxoglutarate docarboxylation in a ligand reaction at the enzyme-bound ferrous ion. Eur. J. Biochem. 138, 239-245 (1984)

7. R. Myllylä, K. Majamaa, V. Günzler, H.M. Hanauske-Abel, and K.I. Kivirikko, Ascorbate is consumed stoichiometrically in the uncoupled reactions catalyzed by prolyl 4-hydroxylase and lysyl hydroxylase. J. Biol. Chem. 259, 5403-5405 (1984)

8. K. Majamaa, T.M. Turpeenniemi-Hujanen, P. Latipää, V. Günzler, H.M. Hanauske- Abel, I.E. Hassinen, and K.I. Kivirikko, Differences between collagen hydroxylases and 2-oxoglutarate dehydrogenase in their inhibition by structural analogues of oxoglutarate. Biochem. J. 229, 127-133 (1985)

9. R.E. Schopf, H.M. Hanauske-Abel, G. Tschank, H. Schulte-Wissermann, and V. Günzler, Effects of hydrazyl group containing drugs on leucocyte functions: An immunoregulatory model for the hydralazine-induced lupus-like syndrome. J. Immunopharmacol. 7, 385-401 (1985)

10. K. Majamaa, V. Günzler, H.M. Hanauske-Abel, R. Myllylä, and K.I. Kivirikko, Partial identity of the 2-oxoglutarate and ascorbate binding sites of prolyl 4-hydroxylase. J. Biol. Chem. 261, 7819-7823 (1986)

11. V. Günzler, H.M. Hanauske-Abel, G. Tschank, and H. Schulte-Wissermann, Immunological effects of thalidomide. Inactivity of the drug and several of its hydrolysis products in mononucleocyte proliferation tests. Arzneimittel-Forsch / Drug Research 36, 1138-1141 (1986)

12. V. Günzler, K. Majamaa, H.M. Hanauske-Abel, and K.I. Kivirikko, Catalytically active ferrous ions are not released from prolyl 4-hydroxylase under turnover conditions. Biochim. Biophys. Acta 873, 38-44 (1986)

13. D.D. Kaska, V. Günzler, K.I. Kivirikko, and R. Myllylä, Characterization of a low-relative-mass prolyl 4-hydroxylase from the green alga Chlamydomonas reinhardii. Biochem. J. 241, 483-490 (1987)

14. V. Günzler, H.M. Hanauske-Abel, R. Myllylä, J. Mohr, and K.I. Kivirikko, Time-dependent inactivation of chick-embryo prolyl 4-hydroxylase by coumalic acid. Evidence for a syncatalytic mechanism. Biochem. J. 242, 163-169 (1987)

15. U. Moebius, V. Günzler, H.M. Hanauske-Abel, E. Spaeth, E. Rüde, and G. Tschank, Butyrate-synchronized cloned T-cells retain their dependence on interleukin-2 for growth induction. Exp. Cell Res. 169, 379-384 (1987)

16. H.M. Hanauske-Abel, G. Tschank, V. Günzler, E. Baader, and P. Gallop, Pyrroloquinoline quinone and molecules mimicking its functional domains. FEBS Lett. 214, 236-243 (1987)

17. G. Tschank, M. Raghunath, V. Günzler, and H.M. Hanauske-Abel, Pyridinedicarboxylates, the first mechanism-derived inhibitors for prolyl 4-hydroxylase, selectively suppress cellular hydroxyprolyl synthesis. Decrease in interstitial collagen and C_{1q} secretion in cell culture. Biochem. J. 248, 625-633 (1987)

18. V. Günzler, H.M. Hanauske-Abel, R. Myllylä, D.D. Kaska, A. Hanauske, and K.I. Kivirikko, Syncatalytic inactivation of prolyl 4-hydroxylase by anthracyclines. Biochem. J. 251, 365-372 (1988)

19. D.D. Kaska, R. Myllylä, V. Günzler, A. Gibor, and K.I. Kivirikko, Prolyl 4-hydroxylase from Volvox carteri. A low-M_r enzyme antigenically related to the alpha -subunit of the vertebrate enzyme. Biochem. J. 256, 257-263 (1988)

20. V. Günzler, D. Brocks, S. Henke, R. Myllylä, R. Geiger, and K.I. Kivirikko, Syncatalytic inactivation of prolyl 4-hydroxylase by synthetic peptides containing the unphysiologic amino acid 5-oxaproline. J. Biol. Chem. 263, 19498-19504 (1988)

21. V. Günzler, Thalidomide - a therapy for the immunological consequences of HIV infection? Med. Hypotheses 30, 105-109 (1989)

22. V. Günzler, H.M. Hanauske-Abel., J.A. Duine, K.I. Kivirikko, and E.J. Corey, Inhibition of collagen hydroxylases by PQQ reveals its domain structure. in PQQ and Quinoproteins (J.A. Jongejan and J.A. Duine, eds), pp. 227-231, Kluwer Academic Publishers, Dordrecht (1989)

23. S. Henke, D. Brocks, H. Gaul, R. Geiger, V. Günzler, H. Plankenhorn, K.I. Kivirikko, and R. Myllylä, Suicide inhibition of prolyl 4-hydroxylase by peptides containing 5- oxa- or 5-azaprolines. in Peptides 1988 (Editors: G. Jung and E. Bayer), pp. 401- 404, Walter de Gruyter, Berlin (1989)

24. G. Kaule and V. Günzler, Assay for 2-oxoglutarate decarboxylating enzymes based on the determination of [1-^{14}C]succinate: Application to prolyl 4-hydroxylase. Anal. Biochem. 184, 291-297 (1990)

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29. V. Günzler, Thalidomide in human immunodeficiency virus (HIV) patients. A review of safety considerations. Drug Safety 7, 116-134 (1992)

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32. R. Myllylä, V. Günzler, K.I. Kivirikko, and D.D. Kaska, Modification of vertebrate and algal prolyl 4-hydroxylases and vertebrate lysyl hydroxylase by diethyl pyrocarbonate. Biochem. J. 286, 923-927 (1992)

33. S.D. Kittur, W.H. Adler, G.R. Martin, M.B. Schapiro, S.I. Rapoport, and V. Günzler, Laminin concentrations in serum and cerebrospinal fluid in aging and Alzheimer's disease. Int. J. Devl Neuroscience 11, 95-99 (1993)

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37. G. Tschank, J. Sanders, K.H. Baringhaus, F. Dallacker, K.I. Kivirikko and V. Günzler, Structural requirements for the utilization of ascorbate analogues in the prolyl 4-hydroxylase reaction. Biochem. J., 300, 75-79 (1994)

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39. E. Baader, M. Bickel, D. Damm, H.H. Donaubauer, H.W. Fehlhaber, H. Grötsch, V. Günzler, V. Teetz, and M. Volz, Interference in clinical laboratory tests, with special regard to the bilirubin assay: Effects of a metabolite of the new prolyl 4-hydroxylase inhibitor, Lufironil. Eur. J. Clin. Chem. Clin. Biochem. 32, 515-520 (1994)

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44. MJ Bahr, K.H.W. Böker, W. Horn, V. Günzler, and M.P. Manns, Serum laminin P1 levels do not reflect critically elevated portal pressure in patients with liver cirrhosis. Hepato-Gastroenterol. 44, 1200-1205 (1997)

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REVIEW

MAMMALIAN OXYGEN SENSING, SIGNALLING AND GENE REGULATION

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Summary

Oxygen is essential to the life of all aerobic organisms. Virtually every cell type is able to sense a limited oxygen supply (hypoxia) and specifically to induce a set of oxygen-regulated genes. This review summarizes current concepts of mammalian oxygen-sensing and signal-transduction pathways. Since the discovery of the hypoxia-inducible factors (HIFs), a great deal of progress has been made in our comprehension of how hypoxia induces the expression of oxygen-regulated genes. The α subunit of the heterodimeric transcription factors HIF-1, 2 and 3 is unstable under normoxia but is rapidly stabilized upon exposure to hypoxic conditions. Following heterodimerization with the constitutively expressed β subunit, HIFs activate the transcription of an increasing number of genes involved in maintaining oxygen homeostasis at the cellular, local and systemic levels.

Key words: dioxin receptor, erythropoietin, haem protein, hypoxia-inducible factor, PAS domain, protein stability.

Oxygen sensing

An adequate supply of oxygen is essential to all higher organisms because it serves as the terminal electron acceptor in mitochondrial oxidative phosphorylation and because several enzymatic processes require molecular oxygen as a substrate. Even slight reductions in normal oxygen concentrations (hypoxia) can cause the induction of specific genes involved in mammalian oxygen homeostasis such as erythropoietin or vascular endothelial growth factor (VEGF). Investigations of such hypoxia-inducible genes performed in many different cultured cell lines suggest that every mammalian (perhaps even every vertebrate and insect) cell possesses one or several oxygen-sensing mechanism(s), i.e. a molecular oxygen sensor. Certain cell types, which can be found in carotid bodies, airway neuroepithelial bodies and pulmonary arteries, have differentiated into specialized oxygen-sensing cells. However, the mammalian cellular oxygen sensor is not known. The beautifully elaborated principles of oxygen sensing in bacteria and yeast (for a review, see Bunn and Poyton, 1996) have still to find their counterparts in higher organisms. In the nitrogen-fixing bacterium *Bradyrhizobium japonicum*, for instance, the oxygen sensor is a histidine kinase termed FixL which, under anaerobic conditions, phosphorylates and thereby activates the transcription factor FixJ. Interestingly, the oxygen-sensing domain of FixL is a so-called PAS domain (see below), a versatile protein fold resembling a left-handed glove that encloses a haem cofactor (Gong et al., 1998). When oxygen is bound to ferrous (FeII) haem, this domain is in the flattened 'off' state, resulting in a change in the protein conformation and inactivation of the kinase domain.

The haem oxygen sensor hypothesis

So far, virtually all proteins capable of binding molecular oxygen contain iron, usually in the centre of a haem moiety. Thus, it came as no surprise when, in 1988, Bunn and co-workers suggested that the mammalian oxygen sensor could be a haem protein (Goldberg et al., 1988). Apart from hypoxia, cobalt (and to a lesser extent nickel and manganese) salts were capable of inducing erythropoietin gene expression with no additional effects under hypoxic conditions, indicating that ferrous haem iron was replaced by the non-oxygen-binding cations. According to the model, this locks the oxygen sensor in the deoxy conformation. Moreover, carbon monoxide inhibited hypoxic but not cobalt-mediated activation of erythropoietin gene expression, in line with the known properties of carbon monoxide binding to haemoglobin. Blockers of haem synthesis abolished both hypoxic and cobalt-dependent erythropoietin induction. Further evidence for the haem hypothesis was provided by the finding that iron chelators, such as desferrioxamine, are also capable of mimicking hypoxia (Ho and Bunn, 1996; Wang and Semenza, 1993b).

Although the basic findings that led to the hypothesis of a haem oxygen sensor responsible for hypoxic erythropoietin induction have been confirmed for many other hypoxia-inducible genes, several discrepancies remain. For example

differential effects of the flavoprotein inhibitor diphenylene iodonium on hypoxia-induced *versus* cobalt-induced gene expression have been reported. Ratcliffe and co-workers found that 50 nmol l^{-1} and 1 μmol l^{-1} diphenylene iodonium inhibited hypoxic but not cobalt- or iron-chelator-dependent induction of several oxygen-regulated genes (Gleadle et al., 1995). In striking contrast, Goldwasser et al. (1995) demonstrated a 50% inhibition of cobalt chloride but not of hypoxic erythropoietin induction by 30 nmol l^{-1} diphenylene iodonium. Schumacker and co-workers reported that diphenylene iodonium and two other mitochondrial inhibitors (rotenone and myxothiazol) blocked hypoxic but not cobalt-dependent induction of erythropoietin mRNA (Chandel et al., 1998). Ratcliffe and co-workers (Gleadle et al., 1995) and Semenza and co-workers (Agani and Semenza, 1998) also showed that the flavoprotein cytochrome P450 reductase inhibitor mersalyl inhibited hypoxia-, cobalt-chloride- and desferrioxamine-dependent induction of the erythropoietin gene. However, mersalyl induced the hypoxia-inducible factor-1α (HIF-1α, see below) and the vascular endothelial growth factor (VEGF) gene (Agani and Semenza, 1998). Mersalyl also induced a reporter gene under the control of transferrin-derived hypoxia-responsive elements (Rolfs et al., 1997) under normoxia without any further effects under hypoxia (R. Wanner and R. H. Wenger, in preparation). Finally, following careful titration of oxygen and cobalt chloride concentrations (taking into consideration that the optimal concentration of the toxic agent cobalt chloride is itself a function of the oxygen concentration), we found that there might well be additive effects on erythropoietin induction when both agents were applied simultaneously (Wenger et al., 1998b).

In summary, these discrepancies suggest either that hypoxia, cobalt and desferrioxamine interact with different steps along the oxygen signal-transduction pathway or that they each affect additional pathways that cross-talk with the oxygen pathway. These contradictory reports also reflect the difficulties of using pleiotropic agents to track down the oxygen sensor to its molecular entity. Different effects of these agents depending on concentration, target genes and cell types have to be considered. Nevertheless, the haem hypothesis is still the best model available. But what kind of haem protein is it?

Many candidates – no election

Because there is no assay available to identify directly the oxygen sensor among the hundreds of known oxygen-binding proteins, many different candidate proteins have been suggested in the past to play this role as a sensor in addition to their known or proposed functions. The following section will provide some examples and discuss briefly the pros and cons for their putative role in oxygen sensing.

One of the most often cited putative haem oxygen-sensing molecules is the cytochrome b_{558}/NADPH oxidase complex that generates superoxide in the plasma membrane of phagocytes and B lymphocytes. The subunits of this complex have also been found in cells of non-immunological origin, including hepatoma cell lines (Görlach et al., 1993, 1994), small cell lung carcinoma (Wang et al., 1996) and pheochromocytoma PC12 cells (Hohler et al., 1999), and in oxygen-sensing cells of the carotid body (the arterial chemoreceptor; Acker et al., 1989; Cross et al., 1990; Kummer and Acker, 1995; Lahiri et al., 1999) and pulmonary neuroepithelial bodies (the airway chemoreceptors; Wang et al., 1996; Youngson et al., 1993, 1997). Therefore, Acker and co-workers put forward the hypothesis that the b_{558} complex may serve as an oxygen sensor (for a review, see Acker, 1994). However, we have demonstrated normal oxygen-regulated gene expression in cell lines derived from patients suffering from chronic granulomatous disease, an inherited disease in which one of the subunits of the b_{558}/NADPH oxidase complex is defective (Wenger et al., 1996). Our conclusion that this complex is not likely to be the 'universal' oxygen sensor has recently been confirmed in knock-out mice deficient in the $gp91^{phox}$ subunit of the b_{558}/NADPH oxidase complex (Archer et al., 1999). Despite a marked reduction in superoxide production, hypoxia caused similar levels of pulmonary vasoconstriction and inhibition of whole-cell K^+ current in both knock-out and wild-type mice.

Another apparently promising candidate oxygen sensor is the principal oxygen-consuming organelle of the cell, the mitochondrion itself. Because the respiratory electron transport chain blocker potassium cyanide cannot induce erythropoietin gene expression, mitochondria were soon ruled out as the oxygen sensor (Goldberg et al., 1988; Tan and Ratcliffe, 1991). Nevertheless, Lahiri and co-workers proposed that mitochondrial cytochrome a_3 (Lahiri et al., 1995; Wilson et al., 1994) and Schumacker and co-workers suggested that mitochondrial cytochrome *c* oxidase (Chandel et al., 1997) could serve as the oxygen sensor. Interestingly, depleting cells of mitochondrial DNA (ρ^0 cells) and treatment with several mitochondrial inhibitors blocked hypoxic induction of oxygen-regulated genes (Chandel et al., 1998). However, because potassium cyanide does not interfere with hypoxic induction of HIF-1α (Jiang et al., 1996), the role of mitochondria in oxygen sensing remains unclear.

Various enzymatic processes that result in the production of different second-messenger molecules are catalyzed by haem proteins. For instance, the guanylate cyclase and cytochrome P450 of the 4A family, producing cyclic GMP (cGMP) and 20-hydroxyeicosatetraenoic acid (20-HETE), respectively, have been proposed as oxygen sensors (Harder et al., 1996; Taylor et al., 1998). Cytochrome P450 (ant)agonists have also been shown to modulate erythropoietin expression (Fandrey et al., 1990), but the involvement of cytochrome P450 could not be confirmed by spectrophotometric analysis (Ehleben et al., 1998).

What next?

As outlined above, the 'comparative approach' to the identification of the oxygen sensor is biased by our limited knowledge of the function and the number of candidate haem proteins. There may be many more candidates since a haem protein cannot be recognized easily from its primary amino

acid sequence. What would be an unbiased 'empirical approach' to this problem? Oxygen binding is not a sufficient criterion for the sensor; it must connect an oxygen-dependent enzymatic activity (many of which are already known) to the regulation of hypoxia-inducible genes. Therefore, only the inactivation of the (unknown) sensor and an analysis of the consequences on gene activation can serve to identify it unambiguously. Ratcliffe and co-workers used hypoxia-response elements to direct the expression of reporter genes. Following random mutagenesis, cells were selected that had lost the ability for oxygen-regulated gene expression. This approach has been validated by the identification of an HIF-1α inactivation mutation (Wood et al., 1998). The oxygen sensor, however, remained unidentified.

Oxygen signalling

The reactive oxygen species dilemma

Reactive oxygen species (ROS) such as superoxide and peroxide are known to serve as signal transducers in several systems (for a review, see Suzuki et al., 1997). Because oxygen is the main component of these molecules, it seems obvious that their concentration depends upon the environmental oxygen concentration. Fandrey, Jelkmann and co-workers reported a close relationship between oxygen and hydrogen peroxide concentrations and rates of erythropoietin production in hepatoma cells (Fandrey et al., 1994). Hydrogen peroxide sequestration could mimic hypoxia (Canbolat et al., 1998) and addition of hydrogen peroxide inhibited hypoxic induction of erythropoietin (Fandrey et al., 1994). This inhibition could be reversed by cobalt chloride and iron chelation (Fandrey et al., 1997). These findings have been confirmed for the oxygen-regulated genes encoding tyrosine hydroxylase (Kroll and Czyzyk-Krzeska, 1998) and several enzymes involved in glucose metabolism (Kietzmann et al., 1996, 1997). This model is also compatible with the idea that the thiol reducing agent *N*-(2-mercaptopropionyl)-glycine and the oxygen radical scavenger 2-acetamidoacrylic acid, but not many other antioxidants, could mimic hypoxia by inducing HIF-1α under normoxic conditions (Chilov et al., 1999; Salceda and Caro, 1997).

Enzymes generating ROS, such as the cytochrome b_{558}/NADPH oxidase complex (see above), have been proposed as the source for superoxide, which is rapidly converted into peroxide by superoxide dismutase. Acker and co-workers suggested a localized Fenton reaction in perinuclear cytosolic granules containing high iron concentrations (Kietzmann et al., 1998; Porwol et al., 1998). The Fenton reaction describes the non-enzymatic reduction of hydrogen peroxide to a hydroxyl anion and the highly reactive hydroxyl radical by oxidation of the ferrous iron. This model would separate the sites of oxygen sensing and the effects of cobalt/iron chelation, and it would explain the differences observed in the function of these agents. According to this hypothesis, normoxic hydroxyl radical concentrations would inactivate HIF-1α, which is relieved from suppression following a decrease in radical concentrations under hypoxic conditions. A prototype factor for such a mechanism is the iron regulatory protein 2, which is oxidized in an oxygen-dependent manner, ubiquitinated and degraded by the proteasomes in iron-replete cells (Iwai et al., 1998).

In striking contrast to these findings, Schumacker and co-workers reported that cardiomyocytes increase their production of ROS following exposure to hypoxia (Duranteau et al., 1998; Vanden Hoek et al., 1998). Antioxidants inhibited this increase in levels of ROS and also blocked hypoxic inhibition of cardiomyocyte contraction. The mitochondrial cytochrome *c* oxidase was suggested as the oxygen sensor that enhanced ROS production in hypoxic cardiomyocytes (Budinger et al., 1998) and hepatocytes (Chandel et al., 1997). Support for this model was provided by using hepatoma cells depleted of functional mitochondria (ρ^0 cells), which showed no induction of ROS under hypoxia and which failed to induce oxygen-regulated gene expression (Chandel et al., 1998).

Taken together, there is no common consensus about whether ROS concentrations decrease or increase following exposure to acute hypoxia. Further experiments determining the kinetics and dependence on oxygen concentration of ROS production will be required to obtain a more definitive picture.

Alternative or no second messengers?

Apart from ROS, other metabolic processes occurring during hypoxia could potentially serve to sense and signal the cellular oxygenation state. A decrease in pH as a consequence of lactate production by anaerobic glycolysis might be responsible for hypoxic gene induction. The hypoxia-induced increase in ferritin production, for instance, might be regulated by iron release from ferritin as a result of acidification of the cytoplasm (Qi et al., 1995). Similarly, hypoxic induction of the p53 tumour suppressor protein has been reported to be dependent on acidosis (Schmaltz et al., 1998). However, a significant decrease in pH requires prolonged or severe hypoxia (anoxia) rather than the short periods of hypoxia sufficient to induce HIF-1α. Indeed, we have observed no effects of altered pH on oxygen-regulated gene expression (A. Rolfs and R. H. Wenger, unpublished observations). In general, it is difficult to understand how signal transducers such as ROS, redox state, pH, ATP, cAMP/cGMP or adenosine could trigger the ubiquitous, sensitive, specific and very rapid hypoxic induction of gene expression. A specific sensor that signals directly to HIF-1 and/or its degradation machinery seems to be more appropriate to fulfil these tasks.

The hypoxia-inducible factors

Compared with the relative lack of information on the oxygen sensor and its signal-transduction pathway, much more is known about the target of these putative signalling mechanisms. In 1995, Semenza and co-workers discovered HIF-1 on the basis of its ability to bind to a hypoxia-response element (HRE) in the 3′ flanking region of the erythropoietin gene (Wang et al., 1995a). HIF-1 is a ubiquitously and constitutively expressed heterodimeric transcription factor

composed of an α subunit unstable under normoxia and a common β subunit, the latter being shared by other transcription factors (for reviews, see Bunn and Poyton, 1996; Semenza, 1998; Wenger and Gassmann, 1997). HIF-1β had previously been identified as the heterodimerization partner of the dioxin receptor/aryl hydrocarbon receptor (AhR) and was hence called AhR nuclear translocator (ARNT, see Hoffman et al., 1991). HIF-1α heterodimerization with ARNT in the nucleus is required for DNA binding and transactivation (Gassmann et al., 1997; Gradin et al., 1996; Kallio et al., 1997; Salceda et al., 1996; Wood et al., 1996) but not for translocation into the nucleus (Chilov et al., 1999; Kallio et al., 1998).

The most intriguing feature of HIF-1α is a so-called oxygen-dependent degradation domain (ODD) that can confer hypoxic stabilization to HIF-1α and to various fusion partner proteins (Huang et al., 1998; Pugh et al., 1997; Srinivas et al., 1999a). Under normoxic conditions, this domain seems to receive a signal that primes the HIF-1α protein for ubiquitination and subsequent degradation in proteasomes. In fact, HIF-1α protein is usually undetectable under normoxic conditions. Proteasomal inhibitors or mutation of the ubiquitin-activating enzyme E1 stabilize the HIF-1α protein but fail to induce oxygen-regulated gene expression (Huang et al., 1998; Kallio et al., 1999; Salceda and Caro, 1997). Normoxic HIF α subunit degradation involves the von-Hippel–Lindau tumour suppressor protein (pVHL) which recognizes and links HIF α subunits, *via* a complex composed of several incompletely characterized proteins, to the ubiquitination machinery (Maxwell et al., 1999). Cells deficient in functional pVHL show constitutively high HIF α subunit levels and expression of many oxygen-regulated genes, leading to haemangioblastoma tumour formation.

The signal that primes the ODD domain for degradation is not known. Reduction–oxidation (redox) reactions might be involved in normoxic HIF-1α degradation, as is the case for other proteins such as iron regulatory protein 2 (Iwai et al., 1998). Certain antioxidants and suppression of the Fenton reaction by iron-chelation can induce HIF-1α protein as well as HIF-1-dependent reporter gene activation (Huang et al., 1996; Salceda and Caro, 1997). However, oxidation of HIF-1α has not been demonstrated so far. Thus, it is not known whether this is a specific mechanism linking HIF-1α to the oxygen sensor or whether the experimental data obtained are the result of nonspecific processes and/or cross-talk with other signal-transduction pathways. It is an intriguing possibility that the autonomous ODD domain of HIF-1α might sense oxygen directly. This hypothesis would predict an iron-containing oxygen-binding domain, either as a haem protein or as an iron–sulphur cluster. However, we (P. Spielmann, F. Parpan and R. H. Wenger, unpublished observations) and others (Srinivas et al., 1999b) could not detect iron in bacterially expressed fragments of HIF-1α that contain the ODD domain.

Although phosphorylation seems to play an important role in the activation of HIF-1α (Salceda et al., 1997; Wang et al., 1995b), replacement of potential phosphoacceptor sites in the critical region of the ODD domain had no effect on its activity (Pugh et al., 1997; Srinivas et al., 1999a). Two recent reports showed that the p42/p44 mitogen-activated protein kinases (MAPKs) can phosphorylate the HIF α subunits, giving it electrophoretic migration properties similar to those of the hypoxically modified protein (Conrad et al., 1999; Richard et al., 1999). However, the phosphorylated amino acid residues have not been identified. MAPK-dependent phosphorylation enhances the transcriptional activity of HIFs, but in non-excitable cells the p42/p44 MAPKs are not activated by hypoxia (Richard et al., 1999). In excitable cells, hypoxia might indirectly activate MAPKs, but HIF α protein stabilization is not related to MAPK-dependent phosphorylation (Conrad et al., 1999). Thus, p42/p44 MAPKs do not account for the most critical step in HIF α subunit activation: the stabilization of the ODD domain.

HIF-1α contains two transactivation (TA) domains, one overlapping with the ODD domain and the second at the C terminus (Huang et al., 1998; Jiang et al., 1997; Li et al., 1996; Pugh et al., 1997). Apart from conferring protein stabilization, these transactivation domains might be further activated under hypoxic conditions (Kallio et al., 1998), for example *via* p42/p44 MAPK-dependent phosphorylation (Conrad et al., 1999; Richard et al., 1999) or redox-dependent processes (Huang et al., 1996; Salceda and Caro, 1997). A reductive environment provided by the redox factor Ref-1 enhances the recruitment of the transcriptional coactivators SRC-1, TIF2 and CBP/p300 (Carrero et al., 2000). Fujii-Kuriyama and co-workers identified a redox-sensitive conserved cysteine residue, mutation of which inhibited the transactivation function (Ema et al., 1999). These authors proposed a model in which reduced thioredoxin translocates into the nucleus of hypoxic cells and transfers the redox signal to Ref-1 which, in turn, interacts with this cysteine. Indeed, overexpression of thioredoxin/Ref-1 has been shown previously to amplify the hypoxic signal (Huang et al., 1996). However, the functional importance of this cysteine residue could not be confirmed in other mutation experiments (O'Rourke et al., 1999), and we did not observe nuclear translocation of thioredoxin under hypoxia (U. Alt and R. H. Wenger, unpublished observations). Therefore, the specificity of thioredoxin/Ref-1 in maintaining HIF-1α in a reduced state as part of the hypoxia-signalling mechanism awaits further clarification.

Apart from the ubiquitously expressed HIF-1α, two other members of this family, HIF-2α (Ema et al., 1997; Flamme et al., 1997; Hogenesch et al., 1997; Tian et al., 1997) and HIF-3α (Gu et al., 1998), have been identified which show a more restricted expression pattern. Functional comparison between HIF-1α and HIF-2α *in vitro* revealed many similarities concerning genomic organization, modular protein structure, hypoxic protein stabilization, heterodimerization, DNA binding and the transactivation function of reporter genes (Ema et al., 1999; Hogenesch et al., 1998; Maxwell et al., 1999; O'Rourke et al., 1999; Wenger et al., 1997; Wiesener et al., 1998). *In vivo* gene targeting (knock-out) experiments in mice showed that HIF-1α is required for mesenchymal cell survival during embryonic development: knock-out animals die around midgestation,

Table 1. *Identified HIF-1 target genes*

Hypoxia-inducible HIF-1 target gene	References
Oxygen transport: erythropoiesis	
Erythropoietin	Firth et al. (1994); Semenza et al. (1994); Wang and Semenza (1993a)
Transferrin (iron transport)	Rolfs et al. (1997)
Transferrin receptor (iron uptake)	Lok and Ponka (1999); Tacchini et al. (1999)
Oxygen transport: angiogenesis and vascular tone	
Vascular endothelial growth factor (VEGF)	Forsythe et al. (1996); Levy et al. (1995); Liu et al. (1995)
Flt-1 (VEGF receptor 1)*	Gerber et al. (1997)
Plasminogen activator inhibitor-1	Kietzmann et al. (1999)
Endothelin-1*	Hu et al. (1998)
Inducible nitric oxide synthase (NO production)	Melillo et al. (1995); Palmer et al. (1998)
Haem oxygenase 1 (CO production)	Lee et al. (1997)
Adrenomedullin	Nguyen and Claycomb (1999)
α_{1B}-Adrenergic receptor	Eckhart et al. (1997)
Anaerobic energy: glycolysis and glucose uptake	
Phosphofructokinase L	Semenza et al. (1994)
Aldolase A	Semenza et al. (1994, 1996)
Glyceraldehyde-3-phosphate dehydrogenase	Graven et al. (1999)
Phosphoglycerate kinase 1	Firth et al. (1994); Okino et al. (1998); Semenza et al. (1994, 1996)
Enolase 1	Semenza et al. (1994, 1996)
Lactate dehydrogenase A	Firth et al. (1995)
Glucose transporter-1	Ebert et al. (1995); Okino et al. (1998)
Negative feedback regulation of HIF-1 function	
p35srj (CBP/p300 antagonist)	Bhattacharya et al. (1999)
Others	
Insulin-like growth factor binding protein-1	Tazuke et al. (1998)
Retrotransposon VL30	Estes et al. (1995)

HIF, hypoxia-inducible factor; CBP/p300, a transcriptional coactivator with histone acetyltransferase activity.

showing cardiovascular malformations and open neural tube defects (Iyer et al., 1998; Kotch et al., 1999; Ryan et al., 1998). Mice containing only one mutant HIF-1α allele develop normally but show impaired physiological responses to chronic hypoxia (Yu et al., 1999). Interestingly, in HIF-2α knock-out embryos, the organ of Zuckerkandl (the major catecholamine source during development) does not produce sufficient amounts of catecholamines, leading to embryonic lethality presumably because of bradycardia (Tian et al., 1998). The expression pattern and functional role of HIF-3α have still to be elucidated.

The PAS family of transcription factors and sensors

Another interesting feature of the HIF α subunits is the presence of PAS domains which can also be found in mammalian ARNT1, 2 and 3 and in their corresponding heterodimerization partners (HIF-1α, 2α, 3α, AhR1, 2 and CLOCK) as well as in several other transcription factors including Per1, 2, Sim1, 2 and NPAS1, 2 and 3 (for a review, see Taylor and Zhulin, 1999). PAS is an acronym standing for the first family members known: *P*er, *A*hR/*A*RNT and *S*im. Most of these PAS proteins have been cloned from mammalian and fish species and from *Drosophila*, where they play important roles in various aspects of development, oxygen homeostasis and circadian rhythm.

Most of the PAS domain proteins described to date have been found in the Bacteria and Archea kingdoms, where they often serve as sensors of oxygen levels, redox state or light levels. Intriguingly, the haem-containing pocket of the FixL oxygen sensor is a PAS domain (Gong et al., 1998), and it is tempting to speculate that the HIF-1α PAS domain displays similar features. However, haem binding to the PAS domain has never been detected, and it is unlikely that HIF-1α functions by itself as an oxygen sensor. In Eukarya, the PAS domain has apparently evolved to a heterodimerization interface that is usually preceded by a basic helix–loop–helix DNA-binding and heterodimerization domain. Whether functionally altered or not, it is astonishing that this domain is conserved in proteins of higher organisms that are functionally related to their predecessors in more primitive species.

Oxygen-regulated gene expression

HIF-1-mediated gene activation

Once activated by hypoxia, HIF-1 binds to the consensus HIF-1 DNA binding site (HBS) A/(G)CGTG present in the hypoxia-response elements (HREs) of many oxygen-regulated genes (for a review, see Wenger and Gassmann, 1997). In the erythropoietin gene, this site can also be constitutively bound

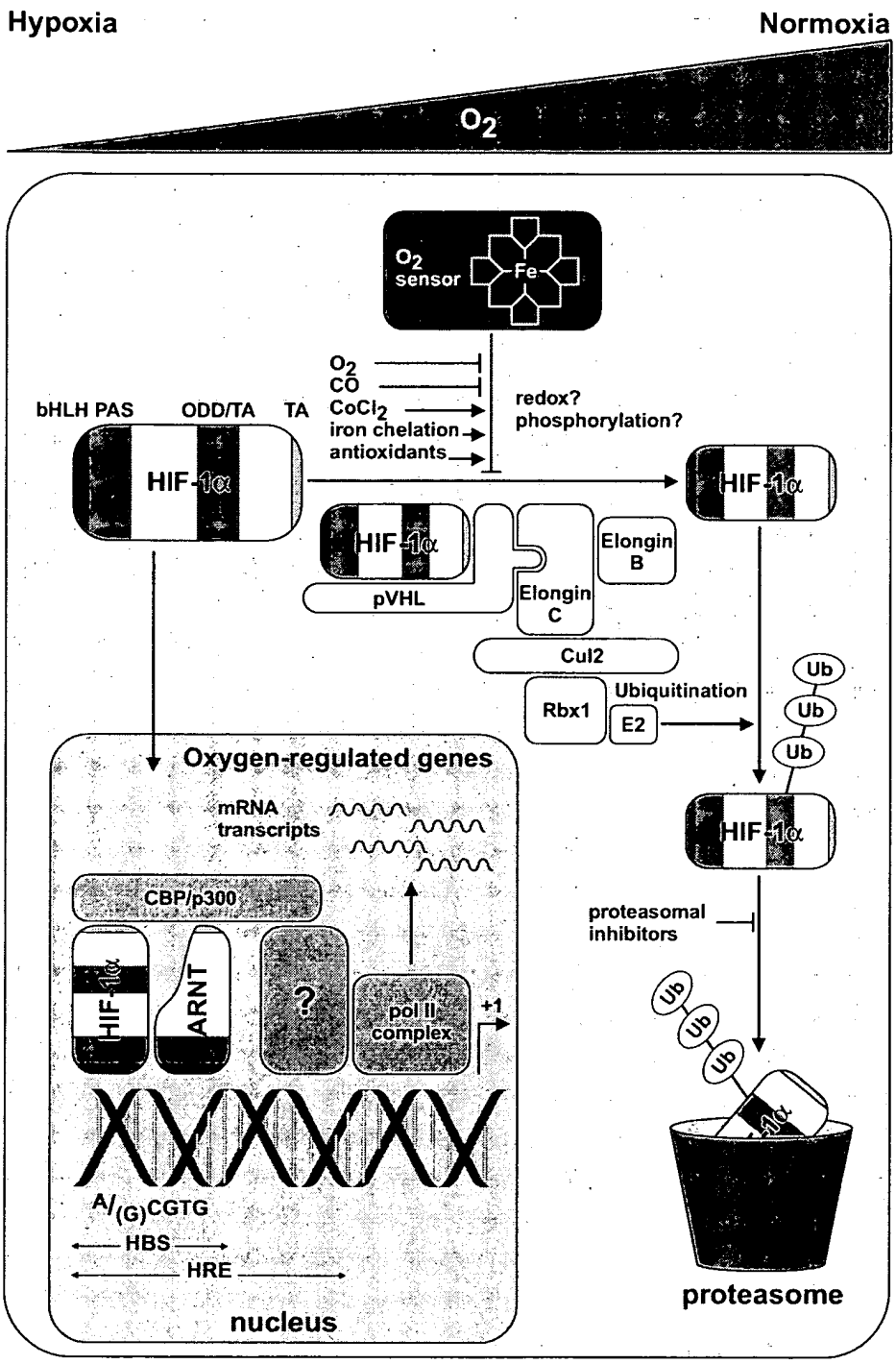

Fig. 1. Hypothetical model of oxygen sensing, signalling and gene regulation. Under hypoxic conditions, the ODD/TA domain of HIF-1α (blue) is stable and allows nuclear translocation and gene regulation. Under normoxic conditions, the ODD/TA domain (orange) obtains a signal from an unknown oxygen sensor which targets HIF-1α for proteolytic degradation via ubiquitination by an incompletely characterized complex containing the pVHL tumour suppressor protein. See text for details. ARNT, aryl hydrocarbon receptor (AhR) nuclear translocator; bHLH, basic-helix-loop-helix; CBP, CREB binding protein (CBP and p300 are two related transcriptional coactivators with histone acetyltransferase activity); Cul2, cullin 2; E2, unknown ubiquitin-conjugating enzyme; HBS, HIF-1 binding site; HRE, hypoxia-response element; ODD, oxygen-dependent degradation domain; PAS, Per-AhR/ARNT-Sim domain (see text); pol II, RNA polymerase II; pVHL, von-Hippel-Lindau tumour suppressor protein; Rbx1, a RING-H2 finger protein; TA, transactivation domain; Ub, ubiquitin; +1 indicates the transcriptional start site.

by the transcription factors activating transcription factor-1 (ATF-1) and cAMP-responsive element-binding-1 (CREB-1) (Kvietikova et al., 1995, 1997). Normoxic occupancy of the HBS (Hu et al., 1997) might protect the CpG dinucleotide present in the HBS from becoming methylation-inactivated (Wenger et al., 1998a). For transactivation, HIF-1 recruits *via*

the two transactivation domains of HIF-1α as well as the C-terminal transactivation domain of ARNT transcriptional coactivators including the histone acetyltransferases CBP/p300 (Arany et al., 1996; Ebert and Bunn, 1998; Ema et al., 1999; Kallio et al., 1998) and SRC-1 and TIF2 (Carrero et al., 2000).

It should be noted that an HBS is necessary, but not sufficient, for efficient gene activation. Binding of other transcription factors in the vicinity of HIF-1 or multimerization of HBSs is usually observed. In the erythropoietin HRE, for example, an unknown factor binding to a CACA box plus the hepatocyte nuclear factor-4 are required to form a functional transcriptional complex (Galson et al., 1995). Other examples include cooperation with the ATF-1/CREB-1 factor in the lactate dehydrogenase A gene (Ebert and Bunn, 1998; Firth et al., 1995) or with activator protein-1 (AP-1) binding factors in the VEGF gene (Damert et al., 1997). Alternatively, we found two adjacent HBSs in the transferrin enhancer (Rolfs et al., 1997), and two or three adjacent HBSs were also found in the genes for phosphoglycerate kinase 1, enolase 1, lactate dehydrogenase A, glucose transporter-1, haem oxygenase 1, p35srj and insulin-like growth factor binding protein-1 (IGFBP-1) (for references, see Table 1).

Several negative feedback regulatory pathways apparently exist to limit the hypoxic response. At the level of mRNA, we found a downregulation of HIF-1α mRNA concentrations by an unknown mechanism following prolonged exposure to hypoxia (Wenger et al., 1998c). At the transactivation level, HIF-1-dependent induction of p35srj has been reported, which in turn binds to the critical coactivator CBP/p300 and blocks the interaction with HIF-1α, resulting in a marked inhibition of transactivation (Bhattacharya et al., 1999).

HIF-1 target genes

Table 1 shows a compilation of the HIF-1 target genes identified so far. Only genes where functional HIF-1 DNA-binding or transactivation has been demonstrated are listed. These genes are involved in oxygen homeostasis at the cellular, local and systemic levels. Erythropoietin activates erythropoiesis to enhance the systemic oxygen transport capacity. Because iron is a limiting factor in haem formation, erythropoiesis is sustained by increased expression of transferrin and transferrin receptor to enhance iron supply to erythroid cells.

At the local level, HIF-1 activates vascular endothelial growth factor, as well as one of its receptors (Flt-1), which induce angiogenesis leading to an increase in the vascular density and hence a decrease in the diffusion distance for oxygen. Local blood circulation is also controlled by modulation of the vascular tone through the production of NO (nitric oxide synthase), CO (haem oxygenase 1), endothelin-1, adrenomedullin or activation of the α_{1B}-adrenergic receptor. It is notable that HIF-1 binding to the HREs of the endothelium-specific genes Flt-1 and endothelin-1 has not been directly demonstrated, and it seems possible that the relatively endothelium-specific HIF-2 might regulate these genes.

At the cellular level, loss of ATP production in mitochondria is compensated by anaerobic glycolysis. Therefore, glucose uptake (glucose transporters) and glycolysis (glycolytic enzymes, see Table 1) are upregulated by HIF-1. Besides hypoxia, both insulin and insulin-like growth factors (IGF)-1 and 2 induce HIF-1α expression (Agani and Semenza, 1998; Feldser et al., 1999; Zelzer et al., 1998). In addition to glucose transporters and glycolytic enzymes, HIF-1 activates IGF-2 and the IGF-binding proteins (IGFBPs) 1, 2 and 3 but not 4, 5 and 6 (Feldser et al., 1999; Tazuke et al., 1998). Other pleiotropic growth factors and cytokines capable of inducing HIF-1 include epidermal growth factor, fibroblast growth factor-2, interleukin-1β and tumour necrosis factor-α (Feldser et al., 1999; Hellwig-Burgel et al., 1999). Thus, these results indicate a complex interplay between positive and negative regulatory growth factors, although further experiments are required to provide a more detailed picture.

Concluding remarks

Fig. 1 summarizes schematically the current models and hypotheses about how a cell senses, signals and reacts to reductions in oxygen concentration. The identification of the oxygen sensor and the corresponding signal-transduction pathway that leads to the activation of HIFs will be of principal research interest for the near future. With regard to the importance of hypoxia in many major diseases (cardiovascular defects, tumour growth), the findings will be of major importance for the treatment of these diseases.

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